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Relationships among the Genera of Pinaceae: An Immunological Comparison

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ABSTRACT. A radioimmunoassay comparison of seed proteins from nine of the ten genera of Pinaceae supports two major groupings in the family. These correspond to those of Van Tieghem, which are also supported by morphological evidence. The abietoid group, which forms a tighter cluster in the immunological analysis, consists of *Abies, Keteleeria, Cedrus, Tsuga,* and *Pseudolarix,* while the pinoid group consists of *Pinus, Picea, Cathaya, Larix,* and *Pseudotsuga.* Vierhapper's classification based on shoot dimorphism is thus rejected as artificial. *Pseudotsuga* and *Larix* form a well marked lineage within the pinoid group as indicated by both immunology and morphology.

The Pinaceae is the largest extant family of conifers, including ten genera and over 200 species (Sporne 1974). It is essentially restricted to the northern hemisphere and has apparently been so throughout its history (Florin 1963). Seven genera occur on two or more continents and have been widely studied. Less well known are *Cathaya, Keteleeria,* and *Pseudolarix,* small genera now restricted to portions of eastern Asia. The family is a natural one and is supported as monophyletic by its protein-type sieve cell plastids (Behnke 1974), pattern of proembryogeny (Dogra 1980), and lack of biflavonoids (Geiger and Quinn 1975). All of these features are unique to the Pinaceae among extant gymnosperms.

The history of the classification of the Pinaceae has been discussed by Florin (1931) and Flous (1936). Many groupings of the genera have been proposed, but two have been most widely used and will be emphasized here.

The classification of Van Tieghem (1891) divided the family into two "groups" (equivalent to subfamilies in the current system) based on the number and position of resin canals in the primary vascular region of the young taproot. The Myélocèles (=abietoid group) included *Tsuga, Cedrus, Abies, Keteleeria,* and *Pseudolarix,* with a single central canal, while the Epixylocèles (=pinoid group), included *Pinus, Picea, Pseudotsuga,* and *Larix,* with resin canals adjacent to each protoxylem pole. Jeffrey (1905) concurred in this classification based on comparative wood anatomy, while Doyle (1945) used it as the base for a diagram of the putative derivation of pollination types. *Cathaya,* described in 1958 by Chun and Kuang, is most similar to the members of the pinoid group in its wood anatomy and cone scale and seed morphology (cf. Chun and Kuang 1962; Greguss 1972) and was recently shown by Hu and Wang (1984) to have the pinoid type of root anatomy.

The second widely used classification is that of Vierhapper (1910), who divided the Pinaceae into two tribes based on occurrence and type of long shoot–short shoot dimorphism. Pineae included only *Pinus,* distinguished on the basis of its unusual short shoots (=needle clusters). Sapineae included the other eight known genera, and was further subdivided into two subtribes. Laricinae included genera with shoot dimorphism (*Pseudolarix, Larix,* and *Cedrus*), while Abietinae included the genera with only long shoots (*Abies, Keteleeria, Tsuga, Picea,* and *Pseudotsuga*). Florin (1963) recognized three subfamilies based on Vierhapper's groupings: Pinoideae (*Pinus*), Abietoideae (Vierhapper's Abietinae plus *Cathaya,* despite weak shoot dimorphism in the latter), and Laricoideae (Vierhapper's Laricinae), and these groupings were adopted by Krüssmann (1972).

More recently, Miller (1976, 1985) has emphasized the division between *Pinus* and the other nine genera based on consideration of certain features of ovulate cone anatomy among extant and fossil members of the family. Thus

TABLE 1. Taxa and seed sources for immunological comparisons. The first six taxa fall into the abietoid group of Van Tieghem (1891) and the latter six into the pinoid group. Abbreviations for seed sources are as follows: Arnold Arboretum, Cambridge, MA (AA); D. P. Fowler (DF), cf. Prager et al. (1976); Inst. of Forest Genetics, Placerville, CA (PL); Maritime Forest Research Centre, Fredericton, New Brunswick (MF); Pacific Forest Research Centre, Victoria, B.C., (PF); Petawawa Forest Expt. Sta., Chalk River, Ontario (CR); Taiwan Forestry Res. Inst., Taipei (TF).

Taxon [Abbrev.]	Source
Abies balsamea (L.) Miller [AbB]	MF
Cedrus deodara (Roxb.) Loudon [CeD]	DF
Keteleeria davidiana (Franchet) Beissner var. *formosana* Hayata [KeD]	TF
Pseudolarix amabilis (J. Nelson) Rehder [PlA]	AA
Tsuga heterophylla (Raf.) Sarg. [TsH]	PF
Tsuga mertensiana (Bong.) Carrière [TsM]	PF
Larix laricina (Du Roi) K. Koch [LaL]	CR
Picea abies (L.) Karsten [PcA]	MF
Picea rubens Sarg. [PcR]	MF
Pinus ponderosa Lawson [PiP]	PL
Pinus strobus L. [PiS]	MF
Pseudotsuga menziesii (Mirbel) Franco [PtM]	PF

there has been considerable disagreement as to the relationships among genera in the Pinaceae based on their morphology.

Prager et al. (1976) conducted an immunological study of seed proteins in representatives of seven genera of the Pinaceae, using Ouchterlony double diffusion. They also used microcomplement fixation to obtain more precise data on a limited subset of the taxa. They were primarily interested in comparing rates of protein evolution and concluded that the seed proteins were changing at a rate comparable to that for intracellular proteins in higher animals, despite slower morphological evolution in the conifers. They did not directly address the question of suprageneric groupings in the family, but presented a Fitch-Margoliash tree consistent with Van Tieghem's classification.

In our current study we have applied a quite different immunological technique, the highly quantitative solid-phase radioimmunoassay (RIA), to corroborate the results of Prager et al. (1976) and explicitly test the classifications of Van Tieghem (1891) and Vierhapper (1910). RIA has been previously applied to phylogenetic comparisons in a variety of animal groups (e.g., Lowenstein et al. 1981; Rainey et al. 1984) and in the chlorophycean algae (Olsen-Stojkovich et al. 1986). It is particularly valuable because it gives quantitative measures of immunological similarity using extremely small amounts of protein and is applicable to groups with a wide range of divergence times. In making phylogenetic inferences from the immunological data one assumes that the average rate of change over a large number of immunological determinants is sufficiently constant as to be approximately proportional to divergence time. This allows an assessment of relationships independent of that from morphology.

In this study we have included representatives of all five of Van Tieghem's abietoid genera and four of the five pinoid genera. Seeds of *Cathaya* are currently unavailable outside of mainland China. The position of the monotypic East Asian genus *Pseudolarix* is of particular interest in that it is placed very differently in the two classifications under consideration. We have also examined and re-analyzed available morphological data for comparison to the immunological results.

MATERIALS AND METHODS

Taxa and seed sources used in the immunological comparisons are listed in table 1. Fully matured germinable seeds of *Pseudolarix amabilis* and *Keteleeria davidiana* were decoated, homogenized, and extracted in isotris buffer (Champion et al. 1974) for four days under rotation. Antisera were prepared using one ml aliquots of the protein-rich supernatant extract injected into New Zealand white rabbits according to the following schedule: primary immunization in Freund's complete adjuvant followed by three secondary injections at two to three week intervals with bleeding at twelve weeks. Antisera and seed extracts for the other taxa were obtained from Prager and Wilson (cf. Prager et al. 1976). All antisera and seed extracts were stored frozen at −10°C.

A "sandwich-technique" RIA (Tsu and Herzenberg 1980; Lowenstein et al. 1981) was used to measure immunological differences among taxa. Because antisera vary in their strength and titer, scout titration assays of homologous reaction pairs were performed in order to deter-

TABLE 2. Cross-reactions of conifer seed proteins. Units are percent uptake of labelled GARGG, with results standardized by rows to give homologous reactions of 10. Rows show reactions to a given antibody and columns to a given antigen. See table 1 for names of taxa.

Anti-body	Antigen											
	KeD	PlA	PiP	PiS	CeD	TsM	TsH	PcR	PcA	LaL	AbB	PtM
KeD	10.0	6.26	4.11	3.68	6.67	5.41	7.69	3.08	2.84	5.47	7.94	4.32
PlA	4.70	10.0	3.57	1.90	3.87	5.33	5.95	3.72	2.53	3.49	4.17	2.17
PiP	3.49	4.07	10.0	8.84	4.44	6.44	3.75	5.74	5.16	6.29	6.23	5.11
PiS	5.04	2.16	4.30	10.0	1.80	2.50	1.63	2.04	2.40	2.50	2.32	3.43
CeD	7.51	5.77	5.46	6.35	10.0	8.04	5.89	4.18	4.59	6.57	10.0	7.50
TsM	4.56	5.16	2.60	3.02	3.36	10.0	5.30	2.98	2.72	4.00	5.46	3.64
TsH	3.26	7.17	4.27	3.60	4.43	9.44	10.0	2.32	2.38	3.51	5.48	4.11
PcR	6.20	4.17	6.73	6.13	3.93	4.67	4.03	10.0	10.0	4.40	4.53	3.60
PcA	4.53	4.70	4.36	4.76	3.20	4.38	2.78	8.32	10.0	4.42	3.81	3.37
LaL	5.57	1.15	5.08	4.59	1.10	4.85	2.27	1.57	1.54	10.0	3.51	5.82
AbB	6.75	2.63	2.33	2.61	3.61	4.19	4.02	1.71	1.71	2.02	10.0	3.38
PtM	4.07	1.56	3.18	3.51	1.82	2.95	1.76	3.68	4.21	6.62	2.78	10.0

mine appropriate reaction concentrations. Optimum antibody concentration is taken from that linear portion of the reaction curve yielding the highest percent binding. Because the antigen phase is bound to the plate, changes in its concentration have less effect. By comparison of a series of homologous reaction curves among different taxa, appropriate dilution adjustments can be made to insure heterologous comparisons are made from the same area of the curves. Both the antigen and homologous antiserum were used for all taxa examined, and all possible antigen-antibody combinations were produced. The resulting immunological distances were standardized using the bindings from the reciprocal homologous reactions.

In the RIA assay, 20 μl of appropriately diluted antigen were bound to each well in polyvinyl microtiter plates (Dynatech Laboratories). After a 1-hr incubation, the unbound material was washed from the plate with 2% bovine serum albumin (BSA), which also blocks further binding to the plate. Twenty μl of appropriately diluted antiserum were then added to each well and allowed to react for 24 hr at room temperature. Unreacted antibody was removed by suction followed by three 2% BSA washes. Finally, 20 μl of a second marker antibody [^{125}I-labelled goat anti-rabbit-gammaglobulin (GARGG)] were added to each well and allowed to incubate for an additional 24 hours. The amount of GARGG bound is proportional to antibody bound in the second step. Excess GARGG was washed out with ten distilled water rinses. The plates were sealed with plastic tape, cut up, and counted for radioactivity on an LKB Gamma Counter.

Immunological distance (ID) was calculated using ID = -100 log Immunological similarity (IS). IS is equal to the sum of the bindings for the reciprocal heterologous reactions divided by the sum for the homologous reactions. Trees were constructed according to the Fitch and Margoliash (1967) algorithm using program FITCH in the program package PHYLIP (version 2.1), written by Joseph Felsenstein (University of Washington, Seattle) and by UPGMA clustering (Sneath and Sokal 1973).

RESULTS

The matrix of binding values for cross-reactions among taxa of Pinaceae is presented in table 2 and the resulting immunological distance matrix in table 3. The values in table 2 are the averaged percent binding of two to three replicate experiments. Reciprocity of cross-reactions (i.e., equivalence of binding values for antigen A vs. antibody B and vice versa) is not always good, but averaging of the reciprocal values tends to compensate for this. Low reciprocity results from complexity of immunological systems, in which antisera and antigens differ in strength and each antiserum reacts with a somewhat different group of antigenic sites. Results of our RIA technique on other study groups (e.g., genera of chlorophycean algae; Olsen-Stojkovich et al. 1986) indicate that rep-

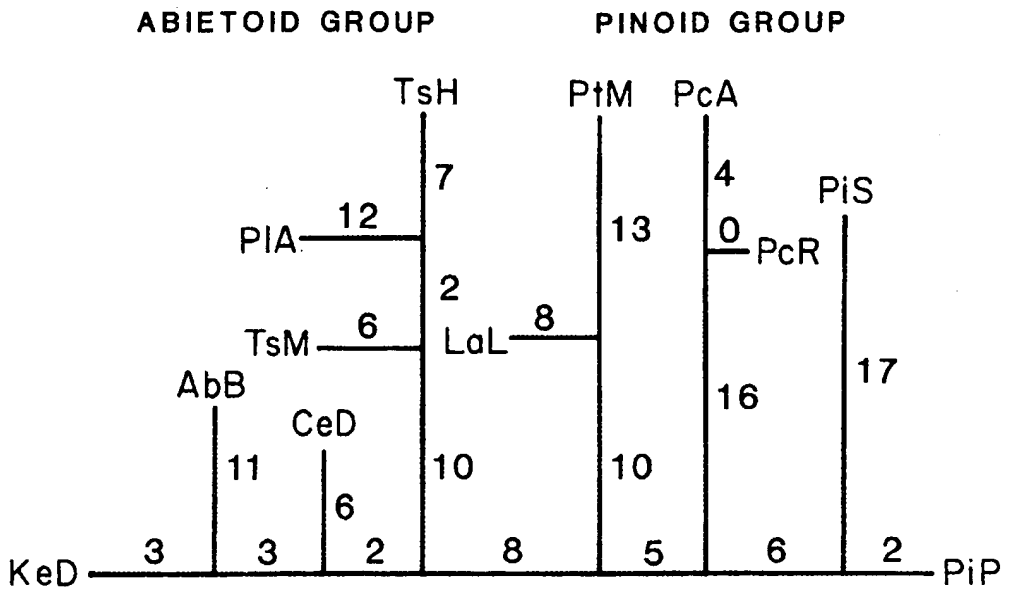

FIG. 1. Unrooted Fitch-Margoliash tree of immunological distances among taxa of Pinaceae. Distances between pairs of taxa are partitioned among nodes so as to minimize "percent standard deviation." See table 1 for names of taxa.

licate antibodies to the same immunizing material raised in different rabbits produce similar results, as do antibodies to different individuals within populations. When pooled antisera are available, reciprocity tends to improve, though it is never perfect.

The "best" tree from the Fitch-Margoliash analysis of the immunological distances is presented in figure 1. This tree gave the lowest percent standard deviation (a measure of goodness of fit of the distances in the cluster diagram to those in the original distance matrix) of 14.74%. Fitch-Margoliash analysis of distances based on the row-row and column-column correlations from the raw bindings (table 2) also gave an identical topology to that in figure 1, indicating that effects of reciprocity are not severe. A UPGMA tree based on the immunological distances is shown in figure 2.

The five genera in Van Tieghem's abietoid

TABLE 3. Immunological distance matrix calculated from table 2.

	Antigen											
Antibody	KeD	PIA	PiP	PiS	CeD	TsM	TsH	PcR	PcA	LaL	AbB	PtM
Keteleeria davidiana	0	26	42	36	15	30	26	33	43	26	13	38
Pseudolarix amabilis		0	42	69	32	28	18	40	44	63	47	73
Pinus ponderosa			0	18	30	34	40	20	32	24	37	38
Pinus strobus				0	39	56	58	39	45	45	61	46
Cedrus deodara					0	24	29	39	41	42	17	33
Tsuga mertensiana						0	13	42	45	35	32	48
Tsuga heterophylla							0	50	59	54	32	53
Picea rubens								0	4	52	51	44
Picea abies									0	53	56	42
Larix laricina										0	56	21
Abies balsamea											0	51
Pseudotsuga menziesii												0

JP

PiS

17

2 PiP

Pinaceae. Distances
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al distance matrix)
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ıhown in figure 2.
'ieghem's abietoid

LaL	AbB	PtM
26	13	38
63	47	73
24	37	38
45	61	46
42	17	33
35	32	48
54	32	53
52	51	44
53	56	42
0	56	21
	0	51
		0

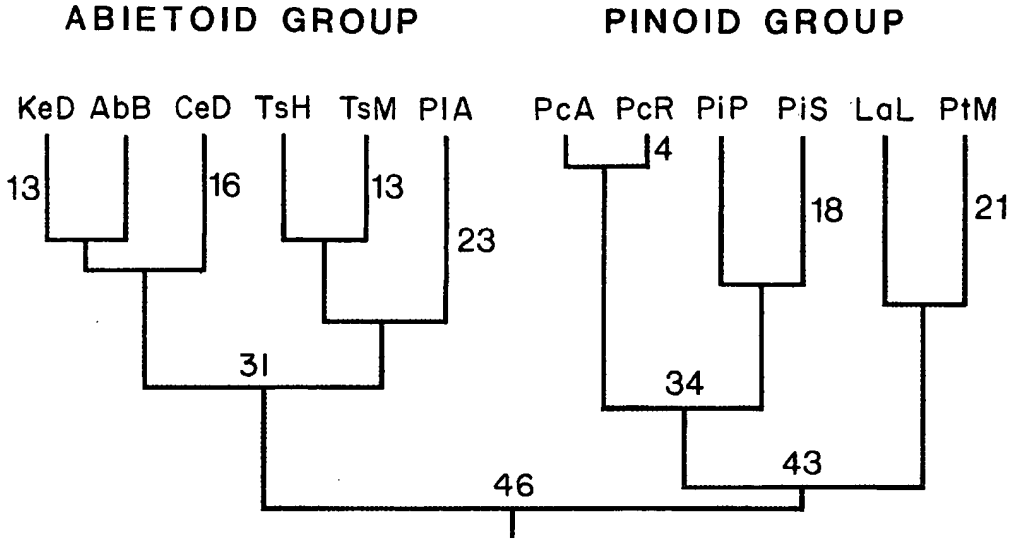

FIG. 2. UPGMA tree of immunological distances among taxa of Pinaceae. See table 1 for names of taxa.

group are placed together in one compact group in our figures 1 and 2, while the four genera examined from the pinoid group are more loosely associated. *Larix* and *Pseudotsuga* are placed on their own lineage within the pinoid group. In our analyses, *Abies*, *Cedrus*, and *Keteleeria* were consistently placed together, but there is some uncertainty as to the order of branching among them. Unsuccessful attempts were made to resolve the trichotomy using absorbed antisera (see Lowenstein et al. 1981 for details of competitive inhibition techniques).

Pseudolarix consistently showed a strong reaction with the *Tsuga* species, but was positioned somewhat differently on the two tree diagrams. In the UPGMA tree (fig. 2), which is based on a more local averaging of distances, the two species of *Tsuga* (representing the two subgenera) are shown as a distinct group adjacent to *Pseudolarix*, while in the FITCH tree (fig. 1), *Pseudolarix* is placed terminal to *Tsuga*. The two species of *Tsuga* are more similar immunologically to one another (ID = 13) than either is to *Pseudolarix* (ID = 18 and 28), so the positioning on the FITCH tree seems less appropriate.

The three pairs of congeneric species included in our analyses provide an internal check on the reliability of the immunological distances. The two species of *Picea*, from the same subgenus, behave almost identically in the immunological comparisons (ID = 4), while the pairs of species from different subgenera of *Pinus* and *Tsuga* are appropriately more distinct (ID = 18 and 13, respectively).

DISCUSSION

Our immunological results place *Pseudolarix* and *Keteleeria* in the abietoid group and thus concur exactly with Van Tieghem's groupings. In contrast, the genera of Vierhapper's Laricinae (*Larix*, *Pseudolarix*, and *Cedrus*) are not shown to be similar immunologically to one another, implying multiple origins and/or losses of shoot dimorphism.

A number of morphological features also support the groupings of Van Tieghem. In addition to the difference in root anatomy noted by Van Tieghem (1891), the five pinoid genera have "normal" resin canals in both the vertical and horizontal systems of their stem wood (Phillips 1948; Hu and Wang 1984), while the abietoid genera have only traumatic resin canals in the stem wood with the apparent exception of the vertical system in *Keteleeria*. The abietoid genera all have seed coats containing resin vesicles, while these are lacking in the pinoid genera (Hickel 1911; Chun and Kuang 1962; confirmed on fresh material by the current authors except for *Cathaya*). It appears that these characters are not logically or function-

ally dependent on one another, and their distribution is quite unlikely to have occurred by chance. Preliminary cladistic analyses of morphological and anatomical features of the Pinaceae (Price et al., unpubl. data) indicate that the abietoid and pinoid groups of Van Tieghem are at least convex (i.e., either both are monophyletic or one gave rise to the other and is thus paraphyletic), while the Laricinae and Abietinae of Vierhapper are polyphyletic.

Within the pinoid group, *Larix* and *Pseudotsuga* are supported as sister groups by our immunological results and those of Prager et al. (1976). Despite their dissimilarity due to the winter-deciduous habit and marked shoot dimorphism of *Larix*, it has long been known that they share a number of morphological features. Both genera have an unusual type of nonsaccate pollen unique among conifers (Erdtman 1965) and a specialized type of micropylar apparatus at the time of pollination (Doyle 1945). Both also have clusters of "fiber-sclereids" in their bark (Chang 1954; Srivastava 1963). All of these features are unique to these two genera within the Pinaceae and are evidently derived features based on outgroup comparison. The two genera also share an unusual karyotype of six isobrachial and six markedly heterobrachial and smaller chromosomes (Khoshoo 1962; Simak 1966; El-Kassaby et al. 1983—*Pseudotsuga menziesii* has an aneuploid derivative of this karyotype with $n = 13$) and have similar seeds and cone scales. Thus the close relationship of these two genera seen in the immunological analysis is well supported by morphological evidence.

The three subgroups seen within the pinoid group (*Pinus, Picea,* and *Larix* plus *Pseudotsuga*) are more distant from one another in our tree diagrams than the genera within the abietoid group. This reflects the average of a number of separate distances and presumably is due to a greater time of divergence. *Pinus* has the longest well established fossil record of the extant genera, dating back to the early Cretaceous (Miller 1976). It is also distinct in a number of characters (e.g., cone scale form and anatomy, and possession of specialized needle clusters). Miller has suggested a major split between *Pinus* and the other extant genera, which would lead to a prediction that *Pinus* should be more distant immunologically from the other genera than they are from one another. This was not observed in our results or those of Prager et al. (1976), which seem to indicate considerable age for *Picea* as well. Further studies with a more diverse sampling of taxa and other macromolecular techniques would be valuable in addressing the question.

Within the abietoid group, *Abies, Keteleeria,* and *Cedrus* are approximately equidistant and quite similar to one another based on the immunological data. Their morphological differences are important enough, however, that they definitely should be retained as separate genera despite their immunological similarity. *Keteleeria* has generally been considered to be most similar to *Abies* or *Pseudolarix* in its morphology (de Ferré and Gaussen 1945; Dallimore and Jackson 1966). It has leaf scars flush to the stem as in *Abies,* umbellate arrangement of male cones as in *Pseudolarix,* and similarities of cone scale and seed morphology to both. Our immunological analysis places *Keteleeria* closest to *Abies* and *Pseudolarix* closest to *Tsuga*. The latter result is unexpected on the basis of morphology and is in need of independent confirmation.

Cedrus has generally been considered most similar to *Abies* based on its ovulate cones, which consist of tightly packed, broadly fan-shaped scales that abscise from the axis at maturity. This accords with their immunological similarity. They also differ in a number of features, however, with *Cedrus* having shoot dimorphism, regular occurrence of ray tracheids in the wood, and broader attachment of the pollen saccae (Phillips 1948; Sivak 1975).

In conclusion, analyses of seed proteins using radioimmunoassay yield groupings identical to those of Van Tieghem (1891), placing *Abies, Keteleeria, Cedrus, Tsuga,* and *Pseudolarix* in one group and *Pinus, Picea, Larix,* and *Pseudotsuga* in another, to which *Cathaya* would be added on the basis of its morphology. Our results using radioimmunoassay are consistent with those obtained from Ouchterlony double diffusion by Prager et al. (1976) and provide a useful check on the methods. Groupings from our immunological trees are concordant with a number of morphological characters and provide a framework for more detailed phylogenetic analysis.

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